

5 **MODULATORS OF BRUTON'S TYROSINE KINASE AND BRUTON'S**
TYROSINE KINASE INTERMEDIATES AND METHODS FOR THEIR
IDENTIFICATION AND USE IN THE TREATMENT AND PREVENTION OF
OSTEOPOROSIS AND RELATED DISEASE STATES.

10 **CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims priority to United States Provisional Patent
Application No. 60/242,471, filed October 23, 2000, and hereby expressly
incorporated by reference in its entirety.

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FIELD OF THE INVENTION

The present invention relates to kinase modulators and methods for their
identification and use in the treatment and prevention of disease. Particularly, the
20 present invention relates to modulators of Bruton's Tyrosine Kinase and Bruton's
Tyrosine Kinase intermediates and methods for their identification and use in the
treatment and prevention of osteoporosis and related disease states.

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BACKGROUND OF RELATED TECHNOLOGY

The osteoclast is a terminally differentiated cell derived from
monocytic/macrophage lineage which resorbs bone as part of the normal process of



5 skeletal modeling and remodeling. In contrast to precursor cells, only fully differentiated mature osteoclasts are able to resorb bone. Increased osteoclastic bone resorption has been linked to the pathogenesis of several skeletal disorders, most notably post-menopausal osteoporosis.

10 As activated osteoclasts move over the bone surface to initiate new sites of bone resorption, cytoskeletal rearrangements lead to the formation of unique cell adhesion structures called podosomes, which attach to the bone matrix via intermediate steps. Podosomes consist of an F-actin core surrounded by the actin-binding proteins vinculin, talin, and α -actinin, and are found in a variety of highly
15 motile cells such as monocytes or macrophages. (Marchisio P.C., et al., *J. Cell Biol.* 99(5):1696-1705 (1984)). Podosome assembly is essential to formation of the sealing zone between osteoclasts and the bone matrix, and subsequent bone resorption by the osteoclast is dependent upon the formation of the sealing zone.

20 It is known that osteoclast precursor cells possess a receptor, receptor activator of NF- κ B (RANK), that recognizes a ligand (RANKL) which leads to osteoclast differentiation. (Suda, T., et al., *Endocr. Rev.*, 20:345-357 (1999)). The RANKL receptor is a member of the tumor necrosis factor (TNF) family and has previously been shown to be an activator of NF- κ B and is a specific inducer of
25 osteoclastogenesis. (Simonet W.S., et. al., *Cell* 89(2):309-319 (1997); Kong Y.Y. et. al., *Nature* 397(6717):315-323 (1999)).

5 Although PI3kinase, rhoA, and pp60c-src have been shown to be essential for
cytoskeletal rearrangement and osteoclast mediated bone resorption, little is known of
the signal transduction events initiated through the RANKL receptor. (Nakamura I., et
al., *J. Cell Physiol.* 172(2):230-239 (1997); Chellaiah M.A., et al., *J. Biol. Chem.*
275(16):11993-20002 (2000); Schwartzberg P.L., et al., *Genes Dev.* 11(21):2835-44
10 (1997)). Several components of the PI3 kinase heteromultimeric complex have been
reported to be responsible for osteoclast activation and bone resorption. In previous
studies, it has been shown that RANKL is a key regulator of osteoclastogenesis and
that the PI3 kinase complex is associated with the RANKL receptor. While it has
been reported that PI3 kinase is involved with ruffled border formation in osteoclasts
15 and that wormannin, a PI3 kinase inhibitor, will affect osteoclast attachment and
spreading leading to subsequent osteopenia, the involvement of BTK in this process
has not been previously demonstrated.

 Additionally, other kinases have been reported to play a role in osteoclast
20 activation. (Matsumoto M., et al., *J. Biol. Chem.* 275, (40) 31155-61 (2000).
However, the link between these kinases, RANKL, and cytoskeletal reorganization
during the activation cycle remains largely unidentified.

 Accordingly, there exists a continuing need to identify compounds involved in
25 the RANKL pathway, as well as modulators thereof, which are useful for the
identification, prevention and treatment of osteoporosis, related disease states and

5 other diseases. The present invention is directed towards meeting these and other needs.

SUMMARY OF THE INVENTION

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It has now been found that Bruton's Tyrosine Kinase (BTK) and intermediates in the BTK pathway are critical intermediates in the cytoskeletal rearrangement pathway leading to osteoclast activation. The present invention further shows that mice deficient in BTK exhibit osteopenia and that this osteopenia can be reversed
15 upon the addition of multiple copies of the BTK gene in transgenic mice. Accordingly, modulators of BTK activity and BTK intermediate activity are useful in affecting osteoclast activation and bone resorption. Such modulators may be identified using assays of the present invention, and are therefore expected to be useful as therapeutic compounds to treat osteoporosis and related disease states. BTK
20 target validation studies on modulators identified using methods of the present invention may be carried out using conventional osteoporosis mouse models. Further, such compounds are suitable for use in compositions for the treatment of osteoporosis and related disease states, and may be administered in any conventional manner. The present invention further includes the use of antisense therapy.

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In one aspect, the present invention is directed to an assay for identifying a compound that modulates the activity of BTK. This assay includes the steps of: (1)

5 providing a cell expressing BTK; (2) contacting the cell expressing BTK with a test compound; and (3) determining whether the test compound modulates the activity of BTK. This assay may be a cell-based assay or may be a cell-free assay, such as a ligand-binding assay. Test compounds which modulate the activity of BTK may be antagonists or agonists, and may bind to BTK. Further, this assay may be used for
10 identifying compounds which will be useful for the treatment of osteoporosis.

In another aspect, the present invention is directed to a method for the treatment of osteoporosis, which includes the step of administering to a patient in need thereof a therapeutically effective amount of a compound identified by the above
15 assay.

In another aspect, the present invention is directed to a method for the treatment of osteoporosis, which includes the steps of: (1) identifying a patient suffering from osteoporosis; and (2) administering to the patient a therapeutically
20 effective amount of a modulator of BTK. The patient may be identified as suffering from osteoporosis by measuring the expression level of BTK in the patient.

In another aspect, the present invention is directed to a method for the prevention of osteoporosis. This method includes the steps of: (1) identifying a
25 patient at risk for osteoporosis; and (2) administering to the patient a therapeutically effective amount of a modulator of BTK. The patient may be identified as being at risk for osteoporosis by measuring the expression level of BTK in the patient.

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In another aspect, the present invention is directed to a method of decreasing the differentiation of osteoclast precursor cells into osteoclast cells. This method includes the step of contacting the osteoclast precursor cells with a BTK modulator.

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In another aspect, the present invention is directed to a compound capable of modulating the activity of BTK. This compound may be identified by the steps of: (1) providing a cell expressing BTK; (2) contacting the cell expressing BTK with the compound; and (3) determining whether the compound modulates the activity of BTK. Such a compound may bind to BTK.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows bone mineral density results for BTK knockout versus wild-type mice.

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Figure 2 shows bone mineral density results for BTK^{xid} mice versus wild-type mice.

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Figure 3 shows the bone mineral density for female BTK^{xid} mice versus wild-type mice with the addition of one and two copies of wild-type BTK on the BTK^{xid} background.

5 Figure 4 shows a summary of molecular constructs generated for studying
BTK.

Figure 5 shows a one-dimensional Western blot showing the detection of
FLAG BTK in transfected COS-7 and HEK 293 lysates.

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Figure 6 shows a one-dimensional Western blot showing the detection of
FLAG BTK in transfected stable RAW 264.7 cell lysates.

15 Figures 7a and 7b show one-dimensional Western blots showing wild-type
BTK and mutant BTK phosphorylation.

Figure 7c shows fluorometric densitometry analysis of anti-flag fluorescence
versus anti phosphotyrosine fluorescence for FLAG tagged BTK and mutants.

20 Figure 8 shows a one-dimensional Western blot showing wild-type BTK and
mutant BTK total cellular tyrosine phosphorylation.

Figure 9 shows immunoprecipitation and kinase assays using SLP 76 as a
kinase substrate.

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Figures 10a, 10b and 10c show actin phalloidin staining of BTK mutant
transfected stable RAW 264.7 cell lines.

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DETAILED DESCRIPTION OF THE INVENTION

The osteoclast is a terminally differentiated cell derived from monocytic/macrophage lineage that resorbs bone as part of the normal process of skeletal remodeling. Increased osteoclastic bone resorption leads to many skeletal disorders, most notably post-menopausal osteoporosis in adult women and frailty in adult men. Through development of podosomes, activated osteoclasts move over the bone surface to initiate new sites of bone resorption. These events are initiated preferentially through the interaction of receptor activator of NF- κ B ligand (RANKL) with the RANKL receptor present on the osteoclast membrane. RAW 264.7 cells may be differentiated into functional osteoclasts upon activation with RANKL. The present invention is directed to the finding that these cells and osteoclasts derived from human and mouse bone tissue have been found to express BTK.

As set forth hereinbelow, in the present invention BTK^{-/-} (Petro J.B., et al., *J. Exp. Med.* 191(10):1745-1754) mice proximal tibia sections evaluated for bone mineral density by peripheral quantitation computed tomography show evidence of osteopetrosis compared to wild-type mice. On the other hand, BTK^{xid} mice (Pinschewer D.D., et al., *Eur. J. Immunol.* 29(9):2981-2987), wherein the mutation results in a conversion of arginine to cysteine at residue 28, are found in the present invention to be osteoporotic compared to wild-type mice. As a result of this mutation,

- 5 the BTK protein is unable to translocate from the cytosol to the inner cell membrane where it subsequently binds to the phospholipid product of PI3 kinase, PIP₃.

Following binding to the phospholipid moiety through the pleckstrin homology domain, BTK is phosphorylated by a membrane associated *src* protein which activates BTK. The activated BTK may then translocate to other subcellular compartments and subsequently regulate other cellular pathways through either its enzymatic activity or association with other regulatory or structural proteins. The osteoporotic effect seen in BTK^{*xid*} mice is reversed by the addition of copies of wild-type BTK transgenes into the BTK/*xid* background. Accordingly, these results of the present invention show that BTK is a critical enzyme in the process of bone resorption and clinical osteoporosis.

Analysis of stable BTK constructs expressed on a RAW 264.7 cell background determined that autophosphorylation of BTK may be inhibited through either the *xid* mutation or the BTK dominant negative mutation (substitution of arginine for lysine at residue 430 in the kinase domain). However, as opposed to the dominant negative mutant as well as the other constructs, the kinase activity of BTK isolated from the *xid* mutant stable cell pool was significantly higher (approximately 10 fold).

Immunofluorescence observations of BTK stable RAW 264.7 cell pools indicated the following differences between mutants stained with actin/phalloidin staining: The dominant negative mutant contained a single ring of podosomes with some stress fibers and cytoplasmic staining; the *xid* mutant contained a double ring of podosomes,

5 irregularly shaped cells with large sealing zones, and cytoplasmic staining; and the
“gain of function” mutation (Li T., et al., *Immunity*. 2(5):451-460) (lysine substituted
for glutamic acid at residue 41 in the pleckstrin homology domain) yielded numerous
large cells containing a significant amount of stress fibers, reminiscent of cytoskeletal
changes observed in RAW 264.7 cells following activation with osteopontin. BTK
10 antibody staining showed localization at or near the membrane regardless of the
mutation. These studies of the present invention establish BTK and subsequent
downstream effectors as critical to podosome assembly and, accordingly, osteoclast
activation and development of osteopenia.

15 The reported DNA sequence (SEQ ID NO:1) and amino acid sequence (SEQ
ID NO:2) of human BTK is set forth in Tables 1 and 2, below, respectively. The
reported DNA sequence (SEQ ID NO:3) and amino acid sequence (SEQ ID NO:4) of
murine BTK is set forth in Tables 3 and 4, below, respectively. Both human and
murine BTK sequences were obtained from the Genbank database.

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One of skill in the art will recognize that BTK suitable for use in the present
invention is desirably murine or human, but may include BTK from any suitable
organism. The protein and genomic sequences of these organisms are readily
accessed via Genbank or The National Center for Biotechnology Information.

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Table 1Nucleotide Sequence of Human BTK: Genbank Accession No. X58957

1 cgtatgtctc cagggccagt gtctgtgctg atcgagtccc acctccaag tcttgccatc
 61 tcaatgcate tgggaagcta cctgcattaa gtcaggactg agcacacagg tgaactccag
 121 aaagaagaag ctatggccgc agtgattctg gagagcatct ttctgaagcg atcccaacag
 10 181 aaaaagaaaa catcacctct aaacttcaag aagcgctgt ttctcttgac cgtgcacaaa
 241 ctctcctact atgagtatga ctttgaacgt gggagaagag gcagtaagaa gggttcaata
 301 gatgttgaga agatcacttg tgttgaaaca gtggttcctg aaaaaaatcc tctccagaa
 361 agacagattc cgagaagagg tgaagagtcc agtgaaatgg agcaaatttc aatcattgaa
 421 aggttccctt atcccttcca ggttgatat gatgaagggc ctctctacgt ctctcccca
 15 481 actgaagaac taaggaagcg gtggttcac cagctcaaaa acgtaatccg gtacaacagt
 541 gatctgggtc agaaatatca ccttgcttc tggatcgatg ggcagtatct ctgctgctct
 601 cagacagcca aaaatgctat gggctgccaa attttgaga acaggaatgg aagcttaaaa
 661 cctgggagtt ctaccggaa gacaaaaag cctctcccc caacgcctga ggaggaccag
 721 atctgaaaa agccactacc gcctgagcca gcagcagcac cagtctccac aagtgcgtg
 20 781 aaaaagggtt tggcccttta tgattacatg ccaatgaatg caaatgatct acagctgcgg
 841 aagggtgatg aatatattat ctggaggaa agcaacttac catggtggag agcacgagat
 901 aaaaatgggc aggaaggcta cattcctagt aactatgtca ctgaagcaga agactccata
 961 gaaatgatg agtggtattc caaacacatg actcggagtc aggtcagca actgctaaag
 1021 caagagggga aagaaggagg ttctattgtc agagactcca gcaaagctgg caaatataca
 25 1081 gtgtctgtgt ttgctaaatc cacaggggac cctcaagggg tgatacgtca ttatgtgtg
 1141 tgtccacac ctacagacca gtattacctg gctgagaagc accttttcag caccatccct
 1201 gagtcatta actaccatca gcacaactct gcaggactca tatccaggct caaatatcca
 1261 gtgtctcaac aaaacaagaa tgcaccttc actgcaggcc tgggatacgg atcatgggaa
 1321 attgatccaa aggacctgac cttctgaag gagctgggga ctggacaatt tggggtagt
 30 1381 aagtatggga aatggagagg ccagtagcac gtggccatca agatgatcaa agaaggctcc
 1441 atgtctgaag atgaattcat tgaagaagcc aaagtcatga tgaatcttc ccatgagaag
 1501 ctggtgcagt tgatggcgt ctgcaccaag cagcgcacca tcttcatcat cactgagtac
 1561 atggccaatg gctgcctcct gaactacctg agggagatgc gccaccgctt ccagactcag
 1621 cagctgctag agatgtgcaa ggatgtctgt gaagccatgg aatacctgga gtcaaagcag
 35 1681 ttcttcacc gagacctggc agctcgaaac tgtttgtaa acgatcaagg agttgttaaa
 1741 gtatctgatt tcggcctgtc caggtatgtc ctggatgatg aatacacaag ctacgtaggc
 1801 tccaaatttc cagtcgggtg gtccccaccg gaagtcctga tgtatagcaa gttcagcagc
 1861 aaatctgaca ttgggcttt tggggttttg atgtgggaaa ttactccct ggggaagatg
 1921 ccatatgaga gatttactaa cagtgcagct gctgaacaca ttgccaagg cctacgtctc
 40 1981 tacaggcttc atctggcttc agagaaggta tataccatca tgtacagttg ttggcatgag
 2041 aaagcagatg agcgtccac ttcaaaaatt cttctgagca atattctaga tgcattgat
 2101 gaagaatcct gagctcgcca ataagcttct tggttctact tctcttctcc acaagcccca
 2161 atttacttt ctacagaggaa atcccaagct taggagccct ggagcctttg tgctccact
 2221 caatacaaaa aggccctct ctacatctgg ggatgcacct ctctttgat tccctgggat
 45 2281 agtggcttct gagcaaggc caaaaaatta ttgtgcctga aatttccga gagaattaag
 2341 acagactgaa ttgcgatga aatatatttt taggagggag gatgtaata gccgcacaaa
 2401 ggggtccaac agctctttga gtaggcattt ggtagagctt gggggtgtgt gtgtgggggt
 2461 ggaccgaatt tggcaagaat gaaatggtgt cataaagatg ggaggggagg gtgttttgat

5 2521 aaaataaatt ctagaaagct taataaaaaa aaaaaaaaaa

Table 2

Amino Acid Sequence of Human BTK: Genbank Accession No. CAA41728

10 1 maavilesif lkrsqqkkkt splnfkkrif lltvhklsyy eydfergrrg skkgsidvek
 61 itcvetvype knppperqip rgeesseme qisiierfpy pfqvvydegp lyvfpsteel
 121 rkrwihqlkn virynsdlvq kyhpcfwidg qylccsqtak namgcqilen rngslkpgss
 181 hrkttkplpp tpeedqilkk plppepaaap vstselkkvv alydympmna ndlqlrkge
 241 yfileesnlp wwrardkngq egyipsnyvt eaedsiemye wyskhmtrsqa aeqllkqegk
 15 301 eggfivrdss kagkytvsfv akstgdpqgv irhyvvcstp qsyyylaekh lfstipelin
 361 yhqhnsagli srlkypvsqq knnapstagl gygsweidpk dltflkelgt gqfgvvkygk
 421 wrgqydvaik mikegsmsed efieeakvmm nlsheklvql ygvctkqrpi fiiteymang
 481 cllnylremr hrfqtqqlle mckdvceame yleskqflhr dlaarnclvn dqgvvkvdsf
 541 glsryvldde ytssvgskfp vrwsppevlm yskfssksdi wafgvlmwei yslgkmpyer
 20 601 ftnsetaehi aqglrlyrph lasekvtyim yscwhekade rptfkillsn ildvmdees

Table 3

Nucleotide Sequence of Murine BTK: Genbank Accession No. L29788

1 aatatgtctc caggccaga gtcttcagag atcaagtcac acctccaag tcctggcacc
 61 tcacgacgtc tggggagcta cctgcattaa gtcagaactg agtacacaaa caagttccag
 10 121 agagaggaag ccatggctgc agtgatactg gagagcatct ttctgaagcg ctcccagcag
 181 aaaaagaaaa catcaccttt aaactcaag aagcgctgt ttctcttgac tgtacacaaa
 241 ctttcatact atgaatatga ctttgaacgt gggagaagag gcagtaagaa aggttcaata
 301 gatgttgaga agatcacctg tgttgaaaca gtaattcctg aaaaaaatcc cccaccagaa
 361 agacagattc cgaggagagg tgaggagtct agtgaaatgg aacagatttc aatcattgaa
 15 421 aggttcccg acccattcca ggtgtatat gatgaaggac ctctctatgt ttctcccca
 481 actgaagage tgagaaagcg ctggattcac cagctcaaaa atgtaatccg gtacaatagt
 541 gacctggtag agaaatacca tccttgcttc tggattgatg gacagtatct ctgctgctct
 601 cagacagcca agaattgctat gggctgcca attttgaga acaggaatgg aagcttaaaa
 661 cctgggagtt ctcacgaaa aacgaaaaag cctcttcccc ctacccaga ggaagatcag
 20 721 atcttgaaaa aaccgcttcc cccggagcca acagcagcac caatctccac aaccgagctg
 781 aaaaagtcgc tggcccttta tgattacatg ccaatgaacg caatgactt acaattgcga
 841 aaggcgagg agtattttat cctggaggag agcaacttac cgtgggtggcg agcacgagat
 901 aaaaatgggc aggaaggcta catcccaagt aactatatca ctgaagctga ggactccata
 961 gagatgatg agtggtattc caagcacatg actcgaagtc aagctgagca actgctaaag
 25 1021 caagagggga aagaaggagg ttctattgtc agagactcca gcaaagctgg aaaatacacc
 1081 gtgtctgtgt ttgctaaatc tactggggag cctcaagggg tgatccgcca ttacgttgtg
 1141 tgttccacgc cacagagcca gtattacctg gctgagaaac acctcttcag caccatccct
 1201 gagtcatta actaccatca acacaactct gcaggcctca tatccaggct gaaatatcct
 1261 gtgtctaaac aaaacaaaaa cgcgccttct actgcaggcc tgggctatgg atcatgggaa
 30 1321 attgatccaa aggacctcac ctcttgaag gagcttggga ctggacaatt cgggtgctgtg
 1381 aaatatggga agtggagggg ccaatatgat gtggccatca agatgatcag agaaggttcc
 1441 atgtcggagg atgaattcat tgaagaagcc aaagtcatga tgaattcttc ccatgagaag
 1501 ctgggtcagt tgtatggcgt ctgcacaaa caacgcccc tcttcatcat caccgagtac
 1561 atggctaagt gctgcctctt gaactacctg agggagatgc ggcaccgctt ccagacacag
 35 1621 cagctgcttg agatgtgcaa agatgtctgt gaagcaatgg aatacttga gtcgaagcag
 1681 ttccttaca gagacctggc agctcgaaac tgttggttaa acgatcaagg agttgtgaaa
 1741 gtatctgact ttggcctgtc taggtatgtc ctgatgatg agtacaccag ctctgtaggc
 1801 tccaagtctc cagtcgggtg gtctccacca gaagtgttta tgtatagcaa gttcagcagc
 1861 aaatctgaca tctgggcttt tggggtttta atgtgggaga tctactccct ggggaagatg
 40 1921 ccgtatgaga gattfactaa cagtgaagca gcagaacaca ttgctcaagg cttacgtctc
 1981 tacaggcttc atctggcacc agagagggtta tatacatca tgtacagctg ctggcacgag
 2041 aaagcagatg aacgtcctag ttcaaaaatt ctcttgagta acattctaga tgtgatggat
 2101 gaagaatcct gagctggctg ctaagctccg tggatctcct cctctctct acaaaaacct
 2161 attcatgtt tctgaggag ttcctggct gcagctctag ctccatgag cctactgaat
 45 2221 gcatgaagag ccttgacat ctaggatgc cttctctc tcgttccctg cgatctgctc
 2281 taagcaagg tcaagggatt tctgtgctta gtattacca taactcaag actcctaaca
 2341 gactgaattg gggacgggaa cactttggg gagggaaaac tgtaaatagc tccactagtt
 2401 gtccaacact tgttggttaa gtgttaagag tgggtggtgt ggtggggggg taggaatgtt

5 2461 gccattaa

Table 4

Amino Acid Sequence of Murine BTK: Genbank Accession No. AAA66943

10 1 maavilesif lkrsqqkkkt splnfkkrif lltvhklsyy eydfergrg skkgsidvek
 61 itcvtvipe knppperqip rgeesseme qisiierfpy pfqvvydegp lyvfspteel
 121 rkrwihqlkn virynsdlvq kyhpcfwidg qylccsqtak namgcqilen rngslkpgss
 181 hrktkkplpp tpeedqilkk plppeptaap isttelkkvv alydympmna ndlqlrkgee
 241 yfileesnlp wwrardkngq egyipsnyit eaedsiemye wyskhmtrs q aeqlkqegk
 15 301 eggfivrdss kagkytvsf akstgepqgv irhyvvcstp qsyyylaekh lfstipelin
 361 yhqhnsagli srlkypvskq nknapstagl gygsweidpk dltflkelgt gqfgvvkygk
 421 wrqgydvaik miregmsed efieeakvmm nlsheklvql ygvctkqrpi fiiteymang
 481 cllnylremr hrfqtqllle mckdvceame yleskqflhr dlaarnclvn dqgvvkvdsf
 541 glsryvldde ytssvgskfp vrwsppevlm yskfssksdi wafgvlmwei yslgkmpyer
 20 601 ftnsetachi aqglrlyrph laservytim yscwhekade rpsfkillsn ildvmdees

Further, derivatives and homologues of BTK may be used in the present invention. For example, nucleic acid sequences encoding BTK of the present invention may be altered by substitutions, additions, or deletions that provide for

25 functionally equivalent-conservative variants of BTK. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of similar properties, such as, for example, positively charged amino acids (arginine, lysine, and histidine); negatively charged amino acids (aspartate and glutamate); polar neutral amino acids; and non-polar amino acids.

30 Other conservative amino acid substitutions can be taken from the Table 5, below.

5

Table 5
Conservative amino acid replacements

For Amino Acid	Code	Replace with any of:
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, β -Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-1-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Other analogs within the invention are those with modifications which

- 10 increase protein stability; such analogs may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the protein sequence. Also included are analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids.

15

5 BTK as used in the present invention may be modified by, for example, phosphorylation, sulfation, acylation, or other protein modifications. It may also be modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

10 It will be apparent to one of skill in the art that conventional screening assays may be used in methods of the present invention for the identification of BTK modulators. By way of example only, one BTK assay suitable for use in the present invention is a BTK Kinase assay set forth hereinbelow under "Materials and Methods". Briefly, this assay may be used to screen for potential BTK inhibitory
15 compounds. The effectiveness of such compounds to inhibit BTK activity may be determined based on decreased SLP 76 phosphorylation. Such compounds may then also be tested for their ability to affect bone resorption *in vitro*.

Further, modulators found to affect BTK activity may further be introduced
20 into a murine osteoporosis model, such as one which has been ovariectomized (which results in a situation similar to postmenopausal osteoporosis), in order to study the ability of such modulators *in vivo*. By way of example only, other murine model systems useful in the present invention for studying bone mass include those described in Matsushita, M., et al., *Am. J. Pathol.*, 125:276-283 (1986) and Kuro-o
25 M., et. al., *Nature*, 390:45-51 (1997).

5 In the present invention, techniques for screening large gene libraries may include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the genes under conditions for detection of a desired activity, e.g., binding of a ligand to BTK in the present invention. Techniques known in the art are amenable to high throughput
10 analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques. High throughput assays can be followed by secondary screens in order to identify further biological activities which will, e.g., allow one skilled in the art to differentiate agonists from antagonists. The type of a secondary screen used will depend on the desired activity that needs to be tested.

15 Drug screening assays are also provided in the present invention. By producing purified and recombinant BTK of the present invention, or fragments thereof, one skilled in the art can use these to screen for drugs which are either agonists or antagonists of the normal cellular function or their role in cellular
20 signaling. In one aspect, the assay evaluates the ability of a compound to modulate binding between BTK of the present invention and a naturally occurring ligand. The term “modulating” encompasses enhancement, diminishment, activation or inactivation of BTK activity. Assays useful to identify ligands to BTK of the present invention, including peptides, proteins, small molecules, and antibodies, that are
25 capable of binding to BTK and modulating its activity, are encompassed herein. A variety of assay formats may be used in the present invention and are known by those

- 5 skilled in the art. One example of a BTK inhibitor is LFM-A13 (Mahajan S., et al., *J. Biol. Chem.* 274(14):9587-99 (1999).

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as primary screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound.

15 Compounds identified using assays, as discussed hereinabove, may be antagonists or agonists of BTK, and may bind to BTK, thereby modulating BTK activity. The term “modulating” encompasses enhancement, diminishment, activation or inactivation of BTK activity. Ligands to BTK of the present invention, including peptides, proteins, small molecules, and antibodies, that are capable of binding to BTK and modulating its activity, are encompassed herein. These compounds are useful in modulating the activity of BTK and in treating BTK-associated disorders.

“BTK-associated disorders” refers to any disorder or disease state in which the BTK protein plays a regulatory role in the metabolic pathway of that disorder or disease. Such disorders or diseases include, but are not limited to, osteoporosis. As used herein the term “treating” refers to the alleviation of symptoms of a particular

- 5 disorder in a patient, the improvement of an ascertainable measurement associated with a particular disorder, or the prevention of a particular immune, inflammatory or cellular response.

A compound which acts as a BTK modulator may be administered for
10 therapeutic use as a raw chemical or may be the active ingredient in a pharmaceutical formulation. Such formulations of the present invention may contain other therapeutic agents as described below, and may be formulated, for example, by employing conventional solid or liquid vehicles or diluents, as well as pharmaceutical additives of a type appropriate to the mode of desired administration (for example,
15 excipients, binders, preservatives, stabilizers, flavors, etc.) according to techniques such as those well known in the art of pharmaceutical formulation.

Compounds of the present invention may be administered by any suitable means, for example, orally, such as in the form of tablets, capsules, granules or
20 powders; sublingually; buccally; parenterally, such as by subcutaneous, intravenous, intramuscular, or intrasternal injection or infusion techniques (e.g., as sterile injectable aqueous or non-aqueous solutions or suspensions); nasally such as by inhalation spray; topically, such as in the form of a cream or ointment; or rectally such as in the form of suppositories; in dosage unit formulations containing non-toxic,
25 pharmaceutically acceptable vehicles or diluents.

5 Such compounds may, for example, be administered in a form suitable for
immediate release or extended release. Immediate release or extended release may be
achieved by the use of suitable pharmaceutical compositions comprising compounds
of the present invention, or, particularly in the case of extended release, by the use of
devices such as subcutaneous implants or osmotic pumps. Compounds of the present
10 invention may also be administered liposomally.

Exemplary compositions for oral administration include suspensions which
may contain, for example, microcrystalline cellulose for imparting bulk, alginic acid
or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and
15 sweeteners or flavoring agents such as those known in the art; and immediate release
tablets which may contain, for example, microcrystalline cellulose, dicalcium
phosphate, starch, magnesium stearate and/or lactose and/or other excipients, binders,
extenders, disintegrants, diluents and lubricants such as those known in the art.

20 Compounds of the present invention may also be delivered through the oral
cavity by sublingual and/or buccal administration. Molded tablets, compressed tablets
or freeze-dried tablets are exemplary forms which may be used. Exemplary
compositions include those formulating the compound(s) of the present invention with
fast dissolving diluents such as mannitol, lactose, sucrose and/or cyclodextrins.

25

Also included in such formulations may be high molecular weight excipients
such as celluloses (avicel) or polyethylene glycols (PEG). Such formulations may

5 also include an excipient to aid mucosal adhesion such as hydroxy propyl cellulose (HPC), hydroxy propyl methyl cellulose (HPMC), sodium carboxy methyl cellulose (SCMC), maleic anhydride copolymer (e.g., Gantrez), and agents to control release such as polyacrylic copolymer (e.g., Carbopol 934). Lubricants, glidants, flavors, coloring agents and stabilizers may also be added for ease of fabrication and use.

10

Exemplary compositions for nasal aerosol or inhalation administration include solutions in saline which may contain, for example, benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, and/or other solubilizing or dispersing agents such as those known in the art.

15

Exemplary compositions for parenteral administration include injectable solutions or suspensions which may contain, for example, suitable non-toxic, parenterally acceptable diluents or solvents, such as mannitol, 1,3-butanediol, water, Ringer's solution, an isotonic sodium chloride solution, or other suitable dispersing or wetting and suspending agents, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

20

Exemplary compositions for rectal administration include suppositories which may contain, for example, a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols, which are solid at ordinary temperatures, but liquify and/or dissolve in the rectal cavity to release the drug.

25

5 Exemplary compositions for topical administration include a topical carrier
such as Plastibase (mineral oil gelled with polyethylene).

The effective amount of a compound of the present invention may be
determined by one of ordinary skill in the art, and includes exemplary dosage amounts
10 for an adult human of from about 0.1 to 100 mg/kg of body weight of active
compound per day, which may be administered in a single dose or in the form of
individual divided doses, such as from 1 to 4 times per day. It will be understood that
the specific dose level and frequency of dosage for any particular subject may be
varied and will depend upon a variety of factors including the activity of the specific
15 compound employed, the metabolic stability and length of action of that compound,
the species, age, body weight, general health, sex and diet of the subject, the mode
and time of administration, rate of excretion, drug combination, and severity of the
particular condition. Preferred subjects for treatment include animals, most preferably
mammalian species such as humans, and domestic animals such as dogs, cats and the
20 like, subject to BTK-associated disorders.

The compounds of the present invention may be employed alone or in
combination with each other and/or other suitable therapeutic agents useful in the
treatment of BTK-associated disorders.

25

In another aspect, the present invention relates to the use of an isolated nucleic
acid in "antisense" therapy. As used herein, "antisense" therapy refers to

5 administration or *in situ* generation of oligonucleotides or their derivatives which specifically hybridize under cellular conditions with the cellular mRNA and/or genomic DNA encoding BTK of the present invention so as to inhibit expression of the encoded protein, e.g., by inhibiting transcription and/or translation. In general, “antisense” therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

Gene constructs useful in antisense therapy may be administered may be administered in any biologically effective carrier, e.g., any formulation or composition capable of effectively delivering a nucleic acid sequence to cells *in vivo*. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenoviruses, adeno-associated viruses, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; an advantage of infection of cells with a viral vector is that a large proportion of the targeted cells can receive the nucleic acid. Several viral delivery systems are known in the art and can be utilized by one practicing the present invention.

In addition to viral transfer methods, non-viral methods may also be employed. Most non-viral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes. Nucleic acid sequences may

- 5 also be introduced to cell(s) by direct injection of the gene construct or by electroporation.

In clinical settings, the gene delivery systems can be introduced into a patient by any of a number of methods, each of which is known in the art. For instance, a
10 pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g., by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination
15 thereof.

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is embedded. Alternatively,
20 where the complete gene delivery system can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

Proteomic analysis of RANKL-induced signal transduction intermediates from
25 RAW 264 cells (murine macrophage cell line) was conducted as set forth in the Examples below. From this analysis, it can be seen that RANKL induces specific tyrosine phosphorylation of BTK, establishing the importance of BTK in the process

- 5 of RANKL-induced osteoclast activation. As such, BTK is an important target in the treatment and prevention of osteoporosis.

The following section sets forth materials and methods used in the present invention, and which were utilized in the Examples set forth hereinbelow.

10

5

MATERIALS AND METHODS

BTK^{-/-} mice: Described in the literature. (Khan W.N., et al., *Immunity* 3(3):283-299). The BTK^{-/-} mice have a mixed genetic background of 129/Sv X C57BL/6. For wild-type controls, 129/Sv X C57BL/6 or C 57BL/6 mice were used.

10

BTK^{xid} mice and BTK^{lo} mice: The BTK transgenic constructs have been described in the literature. (Satterthwaite A.B., et al., *Proc. Natl. Acad. Sci. U S A.* 94(24):13152-13157; Satterthwaite A.B., et al., *Proc. Natl. Acad. Sci. U S A.* 97(12):6687-6692). The BTK^{xid} and BTK^{lo} mice in the present invention are transgenic Balb/C mice derived from the BTK transgenic constructs which contain either the *xid* phenotype or one or two copies of the murine BTK cDNA transgene driven by the Ig heavy chain promoter and enhancer on a BTK *xid* background. The transgene expresses approximately 25% of endogenous BTK protein levels in splenic B cells.

20

Bone Scan: The total and trabecular density of the proximal tibia were evaluated in *ex-vivo* mouse bone samples using an XCT Research SA pQCT (Stratec Medizintechnik, Pforzheim, Germany). The bone was placed in a sample holding tube, and positioned within the gantry of the instrument so that the tibia was in the scanning field. A two-dimensional scout scan was run for a length of 10 mm. After the scout view was displayed on the monitor, the pQCT scan was initiated 1.4 mm distal to the epiphysis. The scan was 1 mm thick, had a voxel (three-dimensional

25

5 pixel) size of 90 μm , and consisted of 180 projections. After the scan was completed, the cross-sectional image was displayed on the monitor. A region of interest was outlined around the tibia. Using an iterative algorithm, soft tissue (density below 223 mg/cm^3) was automatically removed. The density of the remaining bone was reported as total density (mg/cm^3). The outer 55% of the bone was peeled away in a
 10 concentric fashion to determine trabecular density (mg/cm^3).

Generation of recombinant BTK constructs: Primers based on published sequence data (sense: 5'-atacggatccgccgccaccatggctgcagtgcatactg-3' (SEQ ID NO:5), antisense: 5'-tgacgcggccgctcaggattcttcatccatc-3' (SEQ ID NO:6) (Sigma Genosys))
 15 were used to amplify full length murine BTK from Marathon Ready mouse spleen cDNA (Clontech) with Advantaq polymerase (Clontech). Cycling conditions in a Perkin Elmer 9600 thermocycler were as follows: Initial denaturation of 94°C for 2' (minutes), 5 cycles of 94°C for 30', 50°C for 30', 68°C for 2', 5 cycles of 94°C for 30', 55°C for 30', 68°C for 2', 30 cycles of 94°C for 30', 65°C for 30', 68°C for 2',
 20 then 68°C for a 7' extension. The resulting 2kb fragment was isolated from a 1% agarose gel via Quantum Prep Freeze and Squeeze gel purification (BioRad), cloned into PCR2.1TOPO (Invitrogen), electroporated into TOP10 cells (Invitrogen) and spread on LB plates containing 100 $\mu\text{g}/\text{mL}$ ampicillin and X-gal. Individual 5 ml cultures of LB containing 100 $\mu\text{g}/\text{mL}$ ampicillin were inoculated with white colonies
 25 and grown overnight at 37°C with shaking. DNA was obtained (Qiagen robot) and positive clones were selected by restriction enzyme analysis, which was confirmed by sequence analysis. BamHI digested mBTK was cloned into BamHI digested/CIAP

5 treated p3XFLAG-CMV10 expression vector (Sigma). NotI/KpnI digested insert was cloned into NotI/KpnI digested pCDNA 3.1- (Invitrogen).

Generation of recombinant BTK mutants: The Quickchange Site-directed mutagenesis kit (Stratagene) was used to make nucleotide mutations resulting in amino acid changes Arg-28-Cys (XID), Glu-41-Lys (Gain of Function), and Lys-430-Arg (Dominant Negative) in mBTK. Complementary oligonucleotides to the following regions were synthesized and PAGE purified (Sigma-Genosys): R28C: sense- 5'-cctttaaactcaagaagtgcctgttctcttgactg-3' (SEQ ID NO:7), complementary antisense- 5'-cagtcaagagaaacaggcacttctgaagtttaaagg-3' (SEQ ID NO:8) (mBTK nucleotides 57-94), E41K: sense- 5'-ctttcatactataaatatgactttgaacgtggg-3' (SEQ ID NO:9), complementary antisense- 5'-cccacgttcaaagtcattttatagtatgaaag-3' (SEQ ID NO:10) (mBTK nucleotides 102-134), K430R: sense- 5'-ccaatatgatgtggccatcagaatgatcagagaagggttc-3' (SEQ ID NO:11), complementary antisense- 5'-gaaccttctctgatcattctgatggccacatcatattg-3' (SEQ ID NO:12) (mBTK nucleotides 1262-1300).

The oligonucleotide set corresponding to each mutation was annealed to full length mBTK in pcDNA3.1- with Quickchange kit components and cycled in a Perkin Elmer 9600 thermocycler as follows: Initial denaturation of 95°C for 30', 15 cycles of 95°C for 30', 55°C for 1', 68°C for 16', then 68°C for a 1' extension. The methylated parental DNA strand was eliminated by digesting the entire reaction with DpnI for 60' at 37°C. 1 uL was transformed into XL-1 Blue competent cells and plated onto LB

- 5 plates containing 100ug/mL ampicillin. Individual 5 ml cultures of LB containing 100 ug/mL ampicillin were inoculated with colonies and grown overnight at 37°C with shaking. DNA was obtained (Qiagen robot) and mutations verified by sequence analysis. N-terminal FLAG constructs of all mutants were generated by inserting BamHI digested fragments into BamHI digested and CIAP treated p3XFLAG-
- 10 CMV10 expression vector.

- Generation of mBTK pooled stable cell lines: OE6 RAW 264.7 precursor cells were seeded onto 100mm dishes 18 hours prior to transfection. Lipofectamine Plus (Invitrogen/Gibco-BRL) was used for transfection. 4ug Qiagen maxi prep derived
- 15 DNA of all untagged and FLAG-tagged wild-type and mutant mBTK in both pcDNA3.1- and p3XFLAG-CMV 10 were separately combined with 20uL Lipofectamine Plus and 500uL Optimem, incubated at room temperature (RT) for 15' and then combined with a mixture of 500uL Optimem and 30uL Lipofectamine for 15' RT. The mixture was drizzled in a dropwise manner onto the plates in which
- 20 growth media had been replaced with 5 mL Optimem media. Plates were incubated for 3 hours 37°C/5% CO₂ at which time Optimem was removed and replaced with growth medium. 24 hours post-transfection, media was replaced with that containing 900 ug/mL G418.

- 25 Cell culture: RAW 264 cells were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute, Department of Metabolic Diseases, and prepared as follows: Cells were grown in minimal essential media supplemented with 5% fetal

5 bovine serum and 1% nonessential amino acids. For assay purposes, RAW 264 cells were starved for 5 hours in serum free media and then cultured in media containing 2% fetal bovine serum and RANK ligand. When inhibitors were used, the cells were pre-exposed to the inhibitor for one hour prior to RANKL stimulation.

10 Western Blot Analysis of FLAG-BTK Mutants: Confluent RAW 264.7 cells expressing either FLAG vector alone, FLAG-BTK wild-type, FLAG-BTK R28C, FLAG-BTK E41K or FLAG-BTK K430R were washed twice with ice cold PBS and lysed on ice in FLAG-IP lysis buffer (50 mM Tris-HCl [pH=7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1mM sodium orthovanadate, 1X Boehringer protease
15 inhibitor, 1X Sigma phosphatase inhibitor). Lysates were scraped, Dounce homogenized 50 strokes with a tight pestle, transferred to 1.5 ml microfuge tubes, microfuged at 14,000 rpm for 15' and supernatant collected and stored at -80°C. α -FLAG immunoprecipitations were performed on equivalent amounts of lysate in 1 ml total FLAG-IP lysis buffer containing 20 μ l α -FLAG Protein-A Agarose (Sigma).
20 Immunoprecipitations were done with rocking for 2 hours at 4°C, pelleted and washed 4x with TBS. Samples were boiled 3' in Laemmli buffer containing DTT, run on 10% acrylamide Bis-Tris gels (Novex) for 50' at 200 V using MOPS running buffer and blotted onto PVDF for 1 hour at 30 V. Blots were blocked in TBST (TBS + 0.05% Tween) containing 1% BSA for 1 hour at room temperature.

25

Blots were then probed with either α -FLAG-HRP (1:500, UBI) or α -Phosphotyrosine-4G10 (1:2000, UBI) in TBST-BSA for 1 hour at room temperature.

- 5 Blots were then washed 4 times, 5 minutes each, in TBST and either reacted with Amersham ECL+Plus chemiluminescence kit (α -FLAG) or probed with a secondary antibody (α -phosphotyrosine blot probed with α -mouse IgG-HRP, 1:30,000, 1hr, washed 4 times in TBST) and then reacted with ECL+Plus. Bands were visualized using a Fluor-S MAX (Bio-Rad) and quantitations done using Quantity One image
10 analysis software (Bio-Rad).

- IP Kinase Assays: FLAG-BTK wild-type and mutant proteins were immunoprecipitated as described hereinabove under “Western Blot Analysis of FLAG-BTK Mutants.” Immunoprecipitates were washed 3 times with TBS and once
15 with BTK kinase buffer (138 mM NaCl, 50 mM Tris [pH = 8.0], 10 mM MgCl₂, 10 mM MnCl₂). Immunoprecipitates were then resuspended in BTK kinase buffer containing 50 nanograms of purified recombinant SLP-76 as substrate and 40 μ M ATP. Control reactions contained recombinant BTK alone, SLP-76 alone or mock immunoprecipitation (no lysate) with SLP-76. Kinase reactions were carried out for
20 5’ at 37°C, microfuged briefly, supernatants placed on ice, Laemmli buffer added and reactions boiled 3’. Samples were then run on a 10% acrylamide gel, blotted, probed with α -phosphotyrosine, visualized and quantitated exactly as described hereinabove under “Western Blot Analysis of FLAG-BTK Mutants.”

- 25 BTK Kinase Assay and ELISA Protocol:

Materials:

- 5 Sodium Carbonate buffer 0.05 N, pH 8 (Sigma C-3041) for coating the substrate
- Immulon 2 Elisa Plates (Dynatech 0110103455)
- PBS 1x pH 7.5
- Washing buffer (Tween 20 @ .05% final in PBS 1x)
- 10 Blocking buffer [Sanofi Diagnostics blocking buffer 1x (#0220-96)]
- Chromogen mixture = 50% Kirkegaard & Perry labs #50-76-01 TMB
- 50% Kirkegaard & Perry Labs #50-65-00 Peroxidase

Protocol:

- 15 1. Coat Plates with the substrate (GST-LAT full length protein 50 ng/well in 100 µl of Sodium Carbonate buffer. Incubate ON @ 4°C.
2. Wash plates with PBS Tween 20
3. Block Plates with blocking buffer (100 µl/well). Incubate 90 min @ RT.
4. Wash plates with PBS, Flick plate dry
- 20 5. Add 9 ng/well recombinant GST-BTK-KD (Kinase Domain) 100 µl/well in
- kinase buffer (25 mM HEPES pH 7.5, 5 mM MgCl₂, 5 mM MnCl₂, 0.1% BSA, 10 µM ATP).
6. Incubate 60 min 37°C)
- 25 7. Wash plates with PBS Tween 20
8. Add anti-phosphotyrosine mAb 100 µl 1/1000 final dilution (PY99-HRP, Santa Cruz Biotechnology #7020) in blocking buffer (45min @RT)

5 9. Wash as above

10. Add 100 µl/well of Chromogen mixture (Incubate approx. 3-5 min RT)

Quench w/ 100ul 0.1N Sulfuric Acid. Read @ 450/570nm

Total Lysate α-Phosphotyrosine Western Blot: Cells were lysed and 20 µg of

10 total lysate from each sample was electrophoresed, blotted, probed with α-phosphotyrosine and visualized as described hereinabove under “Western Blot Analysis of FLAG-BTK Mutants.”

Immunofluorescence microscopy: FLAG-tagged mBTK wild-type and stable

15 cell line pools were separately seeded at a density of 10E6 cells per 100mm dish each containing collagen I coated overslips (Becton Dickinson). Media was replaced after six hours with media containing 200ng/mL RANK-ligand. On day 5 post-stimulation, media was removed, replaced with 5 mL ice-cold 4% paraformaldehyde/0.1% Triton-X, and the plates incubated at 4°C for 30'. Plates were
20 washed 3X 5 mL ice-cold 1X DPBS/0.1% Triton-X then blocked with 5 mL 1X DPBS/0.1% Triton-X containing 4% non-fat dry milk at 4°C for 60'. Blocking buffer was replaced with 2mL rhodamine-phalloidin (Molecular Probes), diluted 1:40 in DPBS/0.1% Triton-X containing 4% non-fat dry milk, and incubated at 4°C for 10'. Plates were washed 3X 5 mL ice-cold 1X DPBS/0.1% Triton-X. Overslips were
25 each removed and mounted cell slide down, using prolong Antipode mounting media (Molecular Probes), onto glass slides and dried overnight. Rhodamine-phalloidin bound actin was visualized with 530nmDF35 excitation/580DF30 emission filters in a

- 5 Zeus Axioscop 2 microscope. Images were captured with Optronics DEI-750 Acquire software.

Subcellular fractionation: RAW 264.7 cells were washed twice with ice-cold PBS containing 1 mM sodium orthovanadate and lysed for 5 minutes in Triton X-100 lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.5% Triton X-100, 1 mM sodium orthovanadate, 1 mM NaF, 10 µg/ml leupeptin, 1 TIU/ml aprotinin, and 1 mM PMSF on ice. This aliquot represented the cytosolic fraction. For cytoskeletal proteins, remaining cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM NaF, 10 µg/ml leupeptin, 1 TIU/ml aprotinin, and 1 mM PMSF) for 5 minutes on ice. The cytoskeletal proteins were separated by centrifugation at 16,000 x gravity at 4° C for 15 minutes.

Electrophoresis: Isoelectric focusing was carried out in Pharmacia IPG strips, pH 3-10 nonlinear gradient for approximately 150,000 Vhr. Following equilibration for 15 min in 10% glycerol, 50 mM DTT, 2.3% SDS, and 62.5 mM Tris pH 6.5, the IPG strip was layered onto the top of a 10% acrylamide slab gel (1.00 mm thick), and SDS slab gel electrophoresis was carried out for 5 hours at 20 watts/gel. The slab gels were transferred overnight to PVDF membrane which were then were fixed in a solution of 10% acetic acid-40% methanol for 30 min. followed by staining overnight with the fluorescent dye Sypro Ruby Red (Molecular Probes, Eugene, OR) as described in the manufacturer's protocol. Maximal fluorescence incorporation

5 occurred within 4 hours. For Western blots, the PVDF membranes were blocked for >
2 hours with 1 % bovine serum albumin (BSA) (w/v) in 1 % Tween-Tris buffered
saline (TTBS) (v/v), rinsed in TTBS, incubated with primary antibody diluted 1:2,500
in 1% BSA-TTBS for 2 hours, rinsed in TTBS, and incubated with secondary
antibody diluted 1:5,000 in TTBS for 1 hour. The blot was rinsed with TTBS, and
10 treated with ECL (Pharmacia-Amersham Biotech, Piscatawy, NJ). Images were
generated using a BioRad Fluor-S Max imaging system. The images were then
interpreted using PDQuest 6.1 software (BioRad Laboratories Hercules, CA).
Samples were selected for in-gel digestion based upon information obtained from
digital images generated from chemiluminescent stained western blots compared to
15 Sypro fluorescent stained gel images.

Analytical biochemistry and mass spectrometry:

In-gel Digestion: Selected protein spots from Sypro stained membranes were
20 excised and washed twice with water for 15 min. Samples were then dried under
vacuum in a Savant SpeedVac. The samples were then reduced and alkylated and the
gel pieces were washed with 50% acetonitrile: 100 mM ammonium bicarbonate (v/v)
and dried again under vacuum. The gel pieces were then rehydrated with ammonium
bicarbonate containing 12.5 ng trypsin and incubated overnight at 37 ° C. Following
25 digestion, the gel pieces were extracted with 50% acetonitrile: 100 mM ammonium
bicarbonate (v/v) and the supernatants dried under vacuum. The dried material was
resuspended in formic acid for mass spectral analysis.

5

Liquid chromatography-mass spectrometry: Following the extraction of peptides from the gel pieces, the samples were evaporated to dryness (Model AES2010, Savant Instruments, Holbrook, NY). The peptides were dissolved in 5% formic acid, vortexed, sonicated, and then briefly centrifuged to settle insoluble matter. The samples were then loaded onto capillaries packed with a stirred slurry of POROS R2/H (PE-Biosystems, Framingham, MA) using an argon pressure reservoir. The capillaries were pre-equilibrated with >10 column volumes of mobile phase A (A=0.2% isopropanol, 0.1% acetic acid, 0.001% trifluoroacetic acid) prior to the sample loading process. The chromatographic separation was performed with a gradient of increasing organic concentration of 0%B-100%B (B=A+80% acetonitrile) in 45 min at an initial applied pressure of 22 bar generated using a binary HPLC pump (Model 1100, Hewlett-Packard, Palo Alto, CA) flowing at 250 microliters per min. prior to the split. The applied electrospray voltage was 2.2 kV. No sheath gases or make up flows were applied, although the mass spectrometer's heated capillary was operated at 150°C. The sample was sprayed into a Model TSQ7000, (Finnigan, San Jose, CA). The third quadrupole of the mass spectrometer was scanned over the mass to charge range of 475 to 1800 in 1.0 sec. If ions present in this mass range exceeded 80,000 counts, then the three most intense ions present in the spectra were subjected to collision induced dissociation. The collision cell was operated at ~3 mtorr, while the applied collision voltage was adjusted for each precursor ion by multiplying each ion's mass to charge ratio by a factor of 26. The scanned range for the MS/MS scans were also mass to charge dependent, scanning up to a ratio twice that of the precursor

5 ion's apparent mass to charge. The mass spectral data was analyzed by SEQUEST
(ver. 27PVM, Finnigan) on a supercomputer built in-house. The output files were then
each viewed to verify the accuracy of the protein assignments.

As illustrated in the following Examples, it is now found in the present
10 invention that BTK is a critical enzyme in bone resorption and, accordingly, clinical
osteoporosis.

15

Example 1

Effect of Deficiency of BTK Gene on Bone Morphology

The proximal tibial bones of BTK^{-/-} (knockout) mice and their wildtype
counterparts were evaluated to determine the effect of alteration of the BTK gene on
20 bone morphology. The results of the bone mineral density analysis by tomography
shown Figure 1 indicate that tibial sections from BTK^{-/-} mice showed evidence of
osteopetrosis compared to the wild-type mice. These results show that BTK is a
critical enzyme in the process of bone resorption.

25

Example 2

Effect of Mutation of BTK Gene on Bone Morphology

5 The tibial sections from BTK^{xid} mice and their wild-type counterparts were evaluated to determine the effect of the *xid* mutation on bone morphology. The results of the bone mineral density analysis by tomography for these mice is shown in Figure 2 and indicate that tibial section for BTK^{xid} mice showed evidence of osteoporosis compared to the wild-type mice.

10

 This data not only confirms BTK as a key intermediate in the bone resorption process, but also shows that a single point mutation, which would presumably render the protein inactive due to the inability of the BTK to bind to PIP₃, can in fact reverse the observed bone phenotype previously observed with the knockout mice from
 15 osteopetrotic to osteoporotic. To test this possibility, transgenic mice in which copies of the BTK transgene were added back onto the *xid* background were utilized to determine whether a reversal of the osteopenic phenotype was possible. The BTK transgene BTK^{lo} has previously been reported to have an enzymatic activity approximately 25% that of the wild type BTK enzyme. (Satterthwaite A.B., et al., *J.*
 20 *Exp. Med.* 188(5):833-44 (1998)).

 1XTG indicates one copy of the transgene that has approximately 25% wild type level of enzyme and 2X represents two copies of the transgene. Wild-type (wt) plus one or two copies of the transgene would have 100% the normal level of BTK
 25 plus the additional 25% above that for each copy of the transgene present. Therefore, it is possible to have animals with 0, 25, 50, 100, 125 and 150% or wild type levels. In Figure 3, the results from the bone mineral density analysis indicate that tibia from

- 5 BTK *xid* female mice in which the BTK is added back in one or two copies as a transgene, show a trend of increased bone mineral density with the addition of two copies of the normal transgene able to completely compensate for the observed osteopenic *xid* defect.
- 10 These results show that BTK is a critical enzyme in the process of bone resorption, and further show that a single point mutation can reverse the observed bone phenotype previously observed with the knockout mice.

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Example 3BTK Molecular Constructs

Four molecular constructs for overexpression of BTK were designed, and are summarized in Figure 4. Mouse BTK wild-type and three point mutations were cloned into pCDNA 3.1 and p3XFLAG for molecular tagging (3 FLAG epitopes). In the initial construct, wild-type mouse BTK was placed under control of the CMV promoter with a FLAG amino sequence attached to the amino terminal end of the BTK protein coding sequence. Other constructs using the same general design were generated from this construct: (1) the *xid* mutation, which contains a point mutation at residue 28 converting arginine to cysteine; (2) a “gain of function” mutation which was reported in one hemapoetic cell line in which residue 41 was converted from glutamic acid to lysine; and (3) a dominant negative mutation in which the lysine at residue 430 was converted to an arginine, and which is intended to oblivate the kinase activity of the protein.

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Constructs were initially transfected into HEK 293 and COS 7 cell lines. As shown in Figure 5, the FLAG-tagged BTK constructs were successfully expressed in both cell lines. FLAG BTK was detected in transfected COS-7 and HEK 293 lysates with FLAG and BTK COOH terminal 20 amino acid antibodies (detects both FLAG and untagged). These constructs were then transfected into RAW 264.7 cells and cell lysates were examined for expression of FLAG tagged BTK. As shown in Figure 6,

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- 5 transfection and expression into RAW 264.7 cells was successful. FLAG BTK was detected in transfected stable RAW 264.7 cell lysates.

This data establishes that BTK and various constructs thereof may be cloned and stably expressed in a variety of mammalian cell lines. Particularly, BTK
10 constructs may be stably expressed in osteoclast progenitor cell lines.

Example 4

BTK Assays

- 15 Raw 264.7 cells containing the four molecular constructs of BTK were lysed, and the BTK was immunoprecipitated with anti-FLAG antibody. The immunoprecipitated FLAG tagged BTK was Western blotted in duplicate with either anti FLAG antibody or anti-phosphotyrosine antibody and analyzed by chemiluminescence. The mean fluorescence value of the wild-type FLAG tagged
20 BTK was normalized to 100%. As shown in Figure 7a, FLAG BTK was detected in transfected stable RAW 264.7 cell lysates with FLAG antibody. As shown in Figure 7b, phosphotyrosine labeled BTK was detected in the same transfected stable RAW 264.7 cell lysates. Figure 7c shows a fluorometric densitometry analysis of antFLAG fluorescence versus anti-phosphotyrosine fluorescence for FLAG tagged BTK and
25 mutants.

5 As illustrated in Figure 7c, the *xid* BTK phosphorylation is reduced significantly compared to the wild-type BTK, whereas the “gain of function” construct appeared to show a slight increase in BTK tyrosine phosphorylation. The dominant negative construct showed reduced tyrosine phosphorylation. Transfected stable RAW 264.7 cell lysates were then blotted with antiphosphotyrosine to show
10 equivalent levels of total cellular tyrosine phosphorylation, the results of which are shown in Figure 8.

The BTK constructs were then immunoprecipitated from whole cell lysates with anti-FLAG antibody and used to phosphorylate the known BTK substrate, SLP
15 76, in an *in vitro* assay, the results of which are shown in Figure 9. The phosphorylation intensity was monitored by incorporation of phosphotyrosine into the substrate. As shown in Figure 9, recombinant FLAG tagged BTK is able to autophosphorylate itself in the assay, whereas SLP 76 cannot undergo autophosphorylation. In mock and vector alone immunoprecipitations, there is no
20 phosphorylation of the SLP 76 target. However, addition of immunoprecipitated wild-type BTK resulted in phosphorylation of both BTK itself and the SLP 76 target. Addition of BTK immunoprecipitated from the *xid* construct cell lysate resulted in hyperphosphorylation of the SLP 76 target as well as increased phosphorylation of BTK itself. Phosphorylation using extracts from the “gain of function” and dominant
25 negative mutant cell contracts appeared to be reduced compared to the wild-type BTK.

5 These results establish that the BTK^{*xid*} mutation represents a hyperactive form of the enzyme and offers explanation of the role for the *xid* mutation in the establishment of osteopenia in mice. These results further suggests that downstream effector proteins such as, but not limited to, SLP 76 may also contribute to this osteopenic effect by virtue of an increased activity as well as differential

10 compartmentalization of the mutant BTK.

Example 5

Cell Biology

15 Raw 264.7 cells containing the hereinabove stated molecular constructs of BTK were stained with phalloidin and analyzed by fluorescence microscopy. Subtle differences were seen between different mutants stained with actin/phalloidin. As shown in Figure 10a, the wild-type BTK expressing cell showed a single ring of podosomes, some stress fibers, and cytoplasmic staining. As shown in Figure 10b,

20 the R28C (*xid*) showed a double ring of podosomes. These irregularly shaped cells also possessed larger and multiple sealing zones. As shown in Figure 10c, the E41K (“gain of function”) showed numerous large cells containing a lot of stress fibers. BTK Ab showed localization at or near the membrane regardless of mutation. This cell biology data links BTK activation to podosome assembly as well as formation of

25 sealing zones which are necessary structures for subsequent bone resorption by activated osteoclasts.

5 The *xid* form of BTK which has shown evidence of hyperactivity *in vitro* as well as increased osteopenia *in vivo* leads to increased podosome assembly which would in turn lead to enhanced osteoclast activity and subsequent osteopenia. These results also confirm that downstream effector proteins are likely involved in this process leading to osteopenia, particularly downstream effectors and metabolic
10 intermediates which ultimately lead to cytoskeletal reorganization in the activated osteoclast. It should also be apparent that an aberrant BTK protein may also affect transcriptional activity in the osteoclast, as a BTK with an altered pattern of post translational modification is likely to be targeted to a different subcellular compartment. This is further supported by the ability of BTK to activate NF- κ B, a
15 potent modulator of cellular transcriptional activity.

 While the invention has been described in connection with specific embodiments therefore, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the
20 invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims. All references cited herein are expressly incorporated in their entirety.